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Serologic and hexon phylogenetic analysis of ruminant adenoviruses

Howard D. Lehmkuhl · L. A. Hobbs

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Abstract The objectives of this study were to determine the antigenic relationship among ruminant adenoviruses and determine their phylogenetic relationship based on the deduced hexon gene amino acid sequence. Results of reciprocal cross-neutralization tests demonstrated antigenic relationships in either one or both directions among bovine adenovirus type 6 (BAdV-6), BAdV-7, ovine adenovirus type 7 (OAdV-7), caprine adenovirus type 1 (GAdV-1), and deer adenovirus (Odocoileus adenovirus 1, OdAdV-1). No antigenic cross-reactivity was observed among BAdV-1 through -5, and -8 and the other putative ruminant adenoviruses. Two PCR primer sets, one for mastadenovirus and atadenovirus that amplified an approximately 2,700-bp region in the hexon genes were used for comparative studies. Phylogenetic analysis of the deduced hexon amino acid sequences clustered the ruminant adenoviruses on the *Mastadenovirus* and *Atadenovirus* genus branches of the *Adenoviridae* tree. The recent classification of BAdV-6, and -7 as members of the genus *Atadenovirus* was supported by phylogenetic distance matrix analysis of their deduced hexon amino acid sequences. Further, we propose that BAdV-6 and -7 be recognized as members of new *Atadenovirus* species, Bovine adenovirus E and Bovine adenovirus F, respectively. Phylogenetic analysis of OdAdV-1 places this virus in the genus *Atadenovirus* with a proposed new species Odocoileus adenovirus A. OAdV-6 and GAdV-2 are proposed as members of new *Mastadenovirus* species Ovine adenovirus C and Goat adenovirus A, respectively.

H. D. Lehmkuhl (✉) · L. A. Hobbs
Respiratory Disease of Livestock Research Unit,
National Animal Disease Center, Agricultural Research Service,
United States Department of Agriculture, P.O. Box 70,
2300 Dayton Avenue, Ames, IA 50010, USA
e-mail: Howard.Lehmkuhl@ars.usda.gov

Introduction

More than 100 different serotypes of adenoviruses have been isolated from a wide variety of animals. The natural host range is usually confined to either a single species or to closely related animal species. Within host species, adenoviruses are differentiated into serotypes based on immunologic distinctiveness, determined by quantitative cross-neutralization assays. Current literature suggest there are eleven adenovirus serotypes in cattle (BAdV-1 through -10 and Rus) [12, 19], seven in sheep (OAdV-1 through -7) [13], two in goats (GAdV-1 and -2) [24], and one in deer (Odocoileus adenovirus 1, OdAdV-1) [25]. The increased numbers of adenovirus isolates from ruminants and serologic cross-reactivity has made serotyping more cumbersome and problematic due to serologic cross-reactivity observed among putative ruminant atadenoviruses. Quality reagents are often only available in a few reference laboratories to perform the reciprocal cross-neutralization tests necessary to characterize new adenovirus isolates.

Members of the family *Adenoviridae* were originally divided into two genera, *Mastadenovirus* and *Aviadenovirus*, based on isolation from either mammals or birds, respectively, and on the presence of a shared genus-specific antigen detectable by complement fixation, agar gel immunodiffusion, and immunofluorescence. Because some of the BAdVs lacked the *Mastadenovirus* genus-specific antigen and were biologically different, the bovine adenoviruses were divided into two subgroups [4]. Additional

support for division came when genomic analysis demonstrated marked differences in the two bovine subgroups [9]. A new genus, *Atadenovirus*, within in the family *Adenoviridae* was proposed to accommodate this unique adenovirus cluster [10, 14], with acceptance by the International Committee on Taxonomy of Viruses of the International Union of Microbiological Societies, Virology Division, at the 2002 meeting in Paris, France. Ovine adenovirus isolate 287 (OAdV-7) [13], which is antigenically related to BAdV-7, has been designated the prototype species for the genus *Atadenovirus* [12] because it was the first of these unique viruses to have the complete genome sequenced [30]. The subgroup 2 BAdVs (BAdV-4 through -8 and BAdV-Rus) have been assigned to the new genus *Atadenovirus* [11]. Serologic and molecular characteristics of GAdV-1 [23] and OdAdV-1 [25] suggested that these viruses were also members of the genus *Atadenovirus*. The objectives of this study were to determine the antigenic relationship among the ruminant adenoviruses (Table 1) and determine their phylogenetic relationship based on the deduced hexon gene amino acid sequence.

Table 1 Known and putative ruminant members of the family *Adenoviridae*

Virus serotype	Strain	Source	Reference
BAdV-1	No.10	Stormont	Klein et al. [21]
BAdV-2	No. 19	Stormont	Klein et al. [22]
BAdV-3	WBR 1	Stormont	Darbyshire et al. [15]
BAdV-4	THT/62	Stormont	Bartha and Áldásy [5]
BAdV-5	B4/65	Stormont	Bartha and Áldásy [5]
BAdV-6	671130	Stormont	Rondhuis [27]
BAdV-7	Fukuroi	Stormont	Inaba et al. [20]
BAdV-8	Misk/67	Stormont	Bartha et al. [6]
BAdV-10	78–5371	Stormont	Horner et al. [19]
OAdV-1	S1	Stormont	McFerran et al. [26]
OAdV-2	PX515	Stormont	McFerran et al. [26]
OAdV-3	PX611	Stormont	McFerran et al. [26]
OAdV-4	7769	Stormont	Sharp et al. [29]
OAdV-5	SAV	Stormont	Bauer et al. [7]
OAdV-6	WV419/75	Stormont	Davies and Humphreys [16]
OAdV-7	WV/757/75	Stormont	Davies and Humphreys [16]
GAdV-1	NC90-7261	NADC	Lehmkuhl et al. [23]
GAdV-2	T94-0353	NADC	Lehmkuhl et al. [24]
OdAdV-1	D94-2569	NADC	Lehmkuhl et al. [25]

Stormont, Brian M. Adair, Veterinary Science Division, Stormont, Belfast, Northern, Ireland

NADC (National Animal Disease Center), Agricultural Research Service, United States Department of Agriculture, Ames, IA, USA

Materials and methods

Neutralization tests

Antiserum to biologically cloned BAdVs, OAdVs, GAdVs and OdAdV-1 (Table 1) was prepared in rabbits and used in reciprocal serum-virus neutralization tests as previously described [23] to determine the antigenic relatedness of the ruminant adenoviruses. Briefly, serial twofold dilutions of antisera were made starting at 1:32, and viruses were diluted to provide 100 $TCID_{50}$ per well. Controls included back-titration of viruses and a normal rabbit serum negative control. Bovine fetal turbinate cells were used for replication of the BAdVs, ovine fetal turbinate cells for OAdVs, and GAdVs and deer fetal lung cells for replication of OdAdV-1. Virus titers attained in these cell systems ranged from approximately $10^{4.5}$–$10^{6.5}$. Serum antibody titers were expressed as the reciprocal of the highest serum dilution preventing viral CPE in cells of $\geq$50% of the wells after 7 days incubation.

Hexon gene sequence analysis

A PCR primer set that amplified an approximately 2,700-bp internal portion of the atadenovirus hexon gene region was used as described [25]. Additionally, a PCR primer set was developed for members of the genus *Mastadenovirus*. An alignment was made of the 5′- and 3′-end nucleic acid sequences of the hexon genes of three mastadenoviruses (porcine AdV-3 [PAdV-3], GenBank accession #AF083132; BAdV-3, accession #KO1264; and human AdV-3 [HAdV3] accession #X76549) using the ClustalW multiple alignment method (EMBL, Heidelberg, Germany). A consensus-degenerate hybrid oligonucleotide primer strategy [28] was applied to the hexon gene multiple alignment, and primers were designed that would amplify the entire hexon gene except for the final 3′ codon. The primer set (forward primer MaHxF5: 5′-atg gck acs cck tcg atg-3′, reverse primer MaHxR5: 5′-ggt rgc gtt scc ggc bga-3′) was used for mastadenovirus hexon amplification.

Amplification was performed on purified ruminant adenovirus DNA using the Expand Long Template PCR System (Boehringer Mannheim) as described by the system instructions using Buffer 3 and with reaction cycling conditions as follows: 2 min at 94°C; 10 cycles of 94°C for 10 s, 45°C for 30 s, 68°C for 3 min; 20 cycles of 94°C for 10 s, 48°C for 30 s, 68°C for 3 min plus 20 s time extensions; and a final extension at 68°C for 7 min.

An aliquot of the reaction was electrophoresed on 1.0% agarose gel containing 0.1 μg/ml ethidium bromide in TBE (40 mM Tris–borate, 1 mM EDTA, pH 8.0) buffer at 7.5 V/cm for 45 min and visualized by transillumination with ultraviolet light. Multiple bands were produced with the

degenerate primer sets. Bands of the predicted approximate 2,700 bp size were excised and their DNA extracted using the GeneCapsule extraction system (Geno Technology, Inc., St. Louis, MO, USA) and subsequently cloned using the TOPO XL PCR Cloning kit (Invitrogen, San Diego, CA, USA) according to instructions. Transformant plasmid DNA was isolated from *E. coli* and analyzed for the presence of inserts by restriction analysis according to standard techniques [1]. Plasmid DNA was purified for sequencing using the Qiagen Midi Kit (Qiagen, Inc., Chatsworth, CA, USA) according to the manufacturer's instructions. Nucleotide sequences of the cloned bovine, ovine and caprine adenovirus hexon genes were determined by the fluorescent-tagged dideoxynucleotide chain termination method with *Taq*FS polymerase on an Applied Biosystems automated DNA sequencer (ABI, Foster City, CA, USA) with ABI Sequence Analysis Version 2.01 software. Phylogenetic analysis of the deduced ruminant adenovirus hexon gene amino acid sequences and selected published adenovirus hexon gene amino acid sequences was conducted using the ClustalW multiple alignment method (EMBL, Heidelberg, Germany) and the p-distance/neighbor-joining method (MEGA, version 3.1, Kumar, Tamura and Nei, 2004) subjected to 1,000 bootstrap samplings.

Plasmid DNA containing the various adenovirus hexon gene fragments were isolated and analyzed by restriction digestion analysis using *Eco*RI, *Hin*dIII and *Pst*I according to standard and electronic (Mac Vector version 8.0, Mac Vector Inc.) techniques.

Results

There was only homologous neutralization with the BAdV-1 through -6, and -8, OAdV-1 through -6 and GAdV-2 antisera. The results of cross-neutralization tests for the ruminant atadenoviruses are presented in Table 2. Antiserum to BAdV-7 (homologous titer, 8,192) also neutralized OAdV-7, GAdV-1, and OdAdV-1. The OAdV-7 antiserum (homologous titer, 8,192) also neutralized GAdV-1. GAdV-1 antiserum (homologous titer, 32,768) neutralized BAdV-6, -7, OAdV-7, and OdAdV-1. OdAdV-1 antiserum (homologous titer, 262,144) neutralized BAdV-6, -7, OAdV-7, and GAdV-1.

The predicted restriction fragment sizes for *Eco*RI, *Hin*dIII and *Pst*I digests of the cloned ruminant adenoviruses hexon gene amplicons are presented in Table 3. While *Eco*RI digests of BAdV-4, -8 and OAdV-7 did not produce unique patterns, *Hin*dIII digests did. Restriction endonuclease digests with *Eco*RI, *Hin*dIII and *Pst*I did not produce unique patterns with OAdV-1 and -5. Restriction endonuclease patterns generated for the *Eco*RI digest of the atadenovirus cloned hexon gene amplicons are presented in Fig. 1. Nucleotide and predicted amino acid sequence of the near-complete ($\sim$2,700 bp) hexon gene have been filed with GenBank for BAdV-1, (DQ630761); -2, (DQ630762); -5, (AF207658); -6, (AF207659); -7, (AF238232); -8, (AF238233); -10, (AF282774); OAdV-1, (DQ630754); -2, (DQ630755); -3, (DQ630756); -4, (DQ630757); -5, (DQ630758); -6, (DQ630759); GAdV-1, (AF207660); -2, (DQ630760) and OdAdV-1 (AF198354). The results of the phylogenetic analysis of the amino acid sequence alignments of the ruminant adenovirus near-complete hexon genes are shown in Fig. 2. The ruminant adenoviruses segregate to the *Mastadenovirus* and *Atadenovirus* genus branches of the phylogenetic tree. The antigenically related atadenoviruses cluster closely within the genus *Atadenovirus*, with OAdV-7 and GAdV-1 being most closely related. Proposed species assignments are presented in Table 4.

Table 2 Results of cross-neutralization tests between members of the genus *Atadenovirus*

Virus	Immune sera against adenovirus prototype species prepared in rabbits							
	BAdV-4	BAdV-5	BAdV-6	BAdV-7	BAdV-8	OAdV-7	GAdV-1	OdAdV-1
BAdV-4	8,192	–	–	–	–	–	–	–
BAdV-5	–	8,192	–	–	–	–	–	–
BAdV-6	–	–	2,048	–		–	128	512
BAdV-7	–	–	–	8,192	–	–	2,048	128
BAdV-8	–	–	–	–	32,768	–	–	–
OAdV-7	–	–	–	4,096	–	8,192	4,096	256
GAdV-1	–	–	–	4,096	–	8,192	32,768	16,384
OdAdV-1	–	–	–	16,384	–	–	16,384	262,144

Data are expressed as the reciprocals of serum dilutions

NA Not available for inclusion for serological comparisons, – titer $\leq$ 1:32

Table 3 MacVector-predicted restriction fragment size (bp) for ruminant adenovirus hexon gene amplicons cut

BAdV-1	BAdV-2	BAdV-3	BAdV-4	BAdV-5	BAdV-6	BAdV-7	BAdV-8	BAdV-10	0AdV-1	0AdV-2	0AdV-3	0AdV-4	0AdV-5	0AdV-6	0AdV-7	GAdV-1	GAdV-2	OdAdV
*Eco*RI																		
2,724	2,733	2,733	2,733	2,376	2,134	2,022	2,706	1,788	2,724	1,394	2,727	2,730	2,727	2,751	2,736	1,609	2,724	1,366
				330	569	678		954		1,336						1,097		1,364
*Hin*dIII																		
2,293	1,011	2,287	1,536	1,100	1,802	2,534	2,087	1,900	2,724	1,377	1,005	1,798	2,727	2,751	2,001	2,706	2,724	2,558
333	787	446	1,104	987	532	166	532	842		489	787	932			735			93
98	602		93	532	282		87			421	602							79
	333			87	87					333	333							
										110								
*Pst*I																		
2,242	2,539	2,539	2,733	2,706	2,703	2,700	1,622	2,742	2,530	2,536	2,536	1,524	2,533	1,321	1,563	2,706	2,530	2,730
288	194	194					1,084		194	194	194	1,012	194	1,236	732		194	
194												194		194	441			

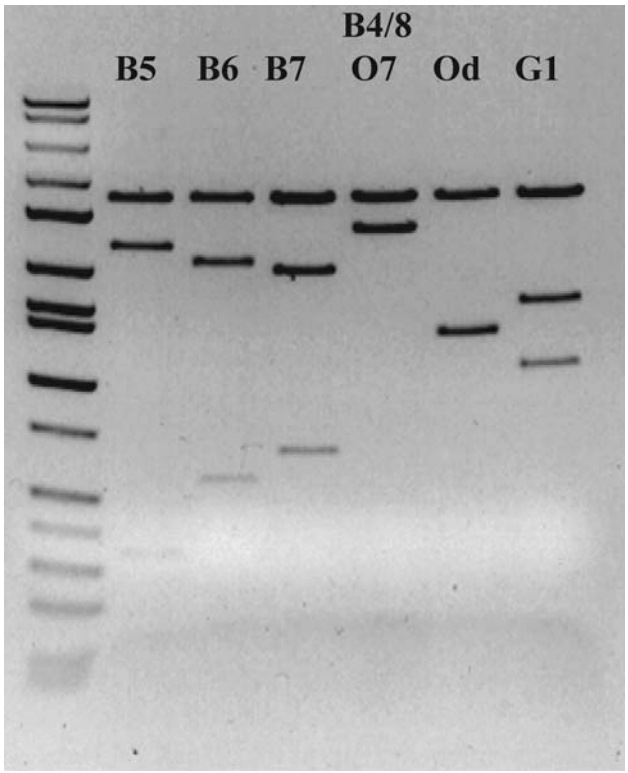

Fig. 1 *Eco*RI digest of cloned PCR amplicons of atadenovirus hexon gene segment for bovine adenovirus type 4 (*B4*) through *B8*, ovine adenovirus type 7 (*O7*), deer adenovirus (*Od*), and caprine adenovirus type 1 (*G1*) separated in a 1% agarose gel, and visualized by ethidium bromide staining. Molecular size standard Hi-Lo™ DNA Marker, Minnesota Molecular, Minneapolis, MN, USA

Discussion

Ruminant adenoviruses cannot be typed based on serology alone. Caution must be exercised when interpreting serum-virus neutralization test results because of demonstrated cross-neutralization between adenoviruses isolated from different ruminant species (Table 2). In characterization studies of U.S. ruminant adenoviruses in our laboratory, we noted serologic cross-reactivity with OAdV-7 (strain ES3048) [18], and OdAdV-1 [25]. Although there is discrepancy with prior publications from our laboratory [18, 23–25], the current information is correct. Unfortunately, there were technical problems with the earlier data. Adenovirus types are defined based on immunological distinctiveness determined by quantitative cross-neutralization assays [12]. A serotype is defined as one devoid of cross-neutralization or that shows a homologous-to heterologous titer ratio greater than 16 in both directions, as is the case with BAdV-1 through -6 and -8, OAdV-1 through -6 and GAdV-2. Where the homologous-to-heterologous titer ratio is 16 or less in one or both directions, as is the case with BAdV-7, OAdV-7, GAdV-1 and OdAdV-1 (Table 2), a new type assignment can be made where

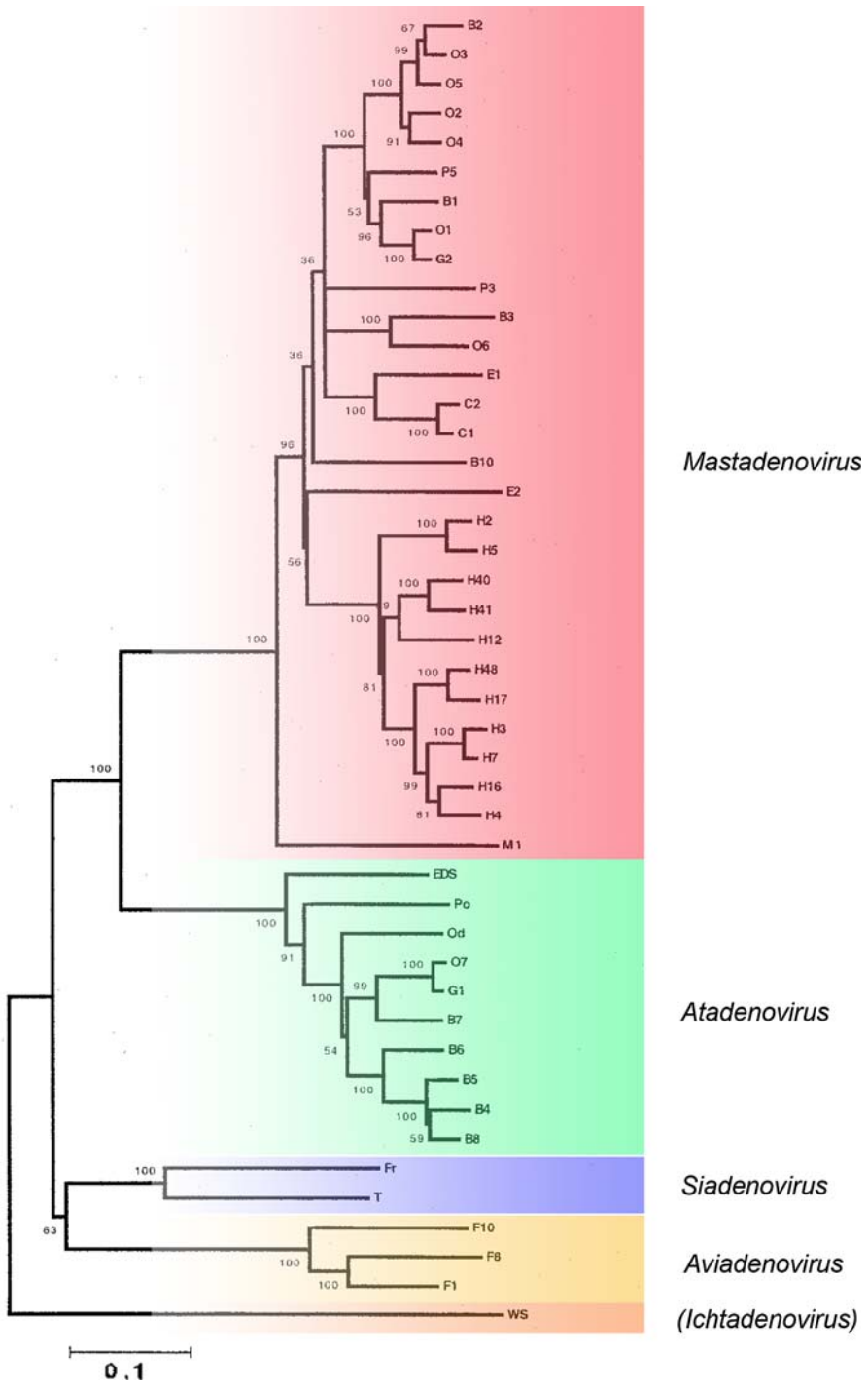

Fig. 2 Unrooted phylogenetic tree of nearly complete hexon gene amino acid sequences of selected adenoviruses (ClustalW multiple alignment method, EMBL, Heidelberg, Germany) generated using the p-distance/neighbor-joining method (MEGA, version 3.1, Kumar, Tamura and Nei, 2004) and subjected to 1,000 bootstrap samplings (bootstrap confidence levels are indicated above each branch). Virus types represented by shortened ICTV abbreviation and GenBank accession numbers for each are as follows: H2 (human AdV-2), P03277; H3, P36849; H4, AAD03660; H5, P04133; H7, P36851; H12, CAA51891; H16, P36854; H17, NC_002067; H40, AAC13967; H41, CAA36079; H48, AAB17439; C1 (canine AdV-1), Q65955; C2, AAB38725; E1 (equine AdV-1), AAB88062; E2, AAB88060; P3 (porcine AdV-3), CAB41030; P5, NC_002702; M1 (murine AdV-1), 6446596; B1 (bovine AdV-1), DQ630761; B2, DQ630762 (NP_597925); B3, HXADB3; B4, AAC41020; B5, AAF20944; B6, AAF20945; B7, AAF63488; B8, AAF638489; B10, AF282774; O1 (ovine AdV-1), DQ630754; O2, DQ630755; O3, DQ630756; O4, DQ630757; O5 DQ630758; O6, DQ630759 O7, AAA84979; G1 (caprine AdV-1), AAF20946; G2, DQ630760; Od (deer AdV-1), AAF13265; EDS (egg drop syndrome virus), CAA70809; F1 (fowl AdV-1), P42671; F8, AAD50344; F10, AAA91647; Po (possum AdV-1), AF338822; Fr (frog AdV-1), AF224336; T (turkey AdV-3), NC_004453; WS (white sturgeon AdV-1), AJ495768

substantial biophysical, biochemical or phylogenetic differences exist. While BAdV-7, OAdV-7, GAdV-1 and OdAdV-1 are closely related antigenically, these viruses are sufficiently distinct culturally and molecularly to justify consideration as new adenovirus types.

Serologic cross-reactivity was previously reported between BAdV-7 and the New Zealand sheep isolate WV757/75 [2]. The homologous-to-heterologous titer ratio was 16 in one direction only. Because there was also a substantial one-way cross-reaction in hemagglutination-inhibition tests, there was insufficient distinction from BAdV-7 for designation as a new sheep adenovirus type. Serologic comparisons of an adenovirus (PI1537/82) isolated from a lamb in Australia and the New Zealand isolate (WV757/75) demonstrated identity between these two isolates [3]. Comparisons of adenovirus isolate PI1537/82 and a second adenovirus from Western Australia (strain 0287) indicated they were identical both serologically and by

Table 4 Adenoviruses of ruminant species

Adenoviridae	
Mastadenovirus	
Bovine adenovirus A	BAdV-1
Bovine adenovirus B	BAdV-3
Bovine adenovirus C	BAdV-10
Ovine adenovirus A	BAdV-2, OAdV-2, -3, -4, -5
Ovine adenovirus B	OAdV-1
Ovine adenovirus C	OAdV-6
Caprine adenovirus A	GAdV-2
Human adenovirus C	BAdV-9
Atadenovirus	
Bovine adenovirus D	BAdV-4, -5, -8, -Rus
Bovine adenovirus E	BAdV-6
Bovine adenovirus F	BAdV-7
Ovine adenovirus D	OAdV-7, GAdV-1
Cervine adenovirus	OdAdV-1

Species names in italic script have been accepted by the International Committee on Taxonomy of Viruses of the International Union of Microbiological Societies, Virology Division. Proposed species are in Roman script

restriction endonuclease analysis [13]. Both were antigenically related to BAdV-7 but were markedly different by restriction enzyme analysis, indicating that these viruses were different and that the ovine isolates should be classified as a new species, Ovine adenovirus 7. Subsequently, 0287 was designated as the prototype strain [11] because the genome is fully sequenced for this virus [30]. Because the New Zealand isolate (WV757/75) was available for serologic comparisons, it was used in the study reported here.

Restriction fragment length polymorphism has been used in the past to assist in classification of BAdV [9] and OAdV [8]. Genome sequence information is available for a wide range of both human and animal adenoviruses. Previous phylogenetic analysis has been based either on the adenovirus protease, hexon, or DNA polymerase gene sequence. The hexon gene was chosen in this study for phylogenetic analysis because the hexon gene is one of the best-characterized adenovirus genes.

The hexon gene PCR primer sets developed for the amplification of the currently recognized ruminant adenoviruses were specific for the *Mastadenovirus* and *Atadenovirus* genera. These are the first primers that amplify the near-full-length adenovirus hexon gene ($\sim$2,700 bp). The sequence generated using our mastadenovirus hexon PCR primer set confirmed the sequence data previously deposited in GenBank for BAdV-2 (accession AC_000001) [17].

Because hexon sequence information is now available in GenBank for most ruminant adenoviruses (BAdV-9 and BAdV-Rus are not available), identification of new ruminant adenovirus isolates can be made by either restriction endonuclease analyses of cloned hexon gene amplicons or sequence alignments, thus eliminating the need for cumbersome reciprocal cross-neutralization tests where quality reagents may not be available and potential confusing cross-neutralization is possible. Sequence alignment analysis to type adenoviruses would be less ambiguous than serology.

An adenovirus type determination could be made for the ruminant adenoviruses using restriction endonucleases *Eco*RI, *Hin*dIII and *Pst*I (Table 3) except for OAdV-1and -5. Restriction endonuclease *Cla*I digests will produce unique fragments, allowing for the differentiation of OAdV-1 and -5.

Phylogenetic analysis of the deduced amino acid sequence of the ruminant adenovirus hexon genes presented here segregates the ruminant adenoviruses into the genera *Mastadenovirus* and *Atadenovirus* (Fig. 2). The antigenically related atadenoviruses cluster closely within the genus *Atadenovirus* with OAdV-7 and GAdV-1 being most closely related. Proposed species assignments are presented in Table 4. There are five proposed new species, two in *Mastadenovirus* and three in *Atadenovirus*.

A phylogenetic tree based upon the hexon amino acid sequences of selected adenoviruses clustered the ruminant adenoviruses in the genera *Mastadenovirus* and *Atadenovirus* (Fig. 2) of the family *Adenoviridae*. This is the first phylogenetic analysis based on the nearly complete hexon gene. The hexon tree includes BAdV-1, OAdV-1 through -6 and GAdV-2, which were not included in any previous hexon tree. Classification of BAdV-6, and -7 as members of the genus *Atadenovirus* based on phylogenetic distance matrix analysis of their protease and short hexon amino acid sequence was confirmed by phylogenetic distance matrix analysis of their deduced nearly complete hexon amino acid sequence and belong to the proposed new *Atadenovirus* species Bovine adenovirus E and Bovine adenovirus F, respectively. Additionally, phylogenetic analysis of OdAdV-1 places this virus in the genus *Atadenovirus* with a proposed new species, Cervine adenovirus A, and OAdV-6 and GAdV-2 are proposed to belong to the tentative *Mastadenovirus* species Ovine adenovirus C and Caprine adenovirus A. Further, the phylogenetic tree presented here suggests that *Ovine adenovirus A* may actually represent two species, one containing OAdV-2 and -4 and the other BAdV-2 and OAdV-3 and -5. Further analysis will need to be done to determine if this is the case.

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